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Alterations in the $\alpha_2\delta$ ligand, thrombospondin-1, in a rat model of spontaneous absence epilepsy and in patients with idiopathic/genetic generalized epilepsies

*¹Ines Santolini, *¹Roberta Celli, *Milena Cannella, *Tiziana Imbriglio, †Michela Guiducci, †Pasquale Parisi, ‡Julian Schubert, §Michele Iacomino, §Federico Zara, ‡Holger Lerche, ²EuroEPINOMICS CoGIE Consortium, ²Genetic Commission of Italian League Against Epilepsy (LICE), *Slavanka Moyanova, ¶Richard Teke Ngomba, #Gilles van Luijtelaaar, *Giuseppe Battaglia, ***Valeria Bruno, ††Pasquale Striano , and ***Ferdinando Nicoletti

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SUMMARY

Objectives: Thrombospondins, which are known to interact with the $\alpha_2\delta$ subunit of voltage-sensitive calcium channels to stimulate the formation of excitatory synapses, have recently been implicated in the process of epileptogenesis. No studies have been so far performed on thrombospondins in models of absence epilepsy. We examined whether expression of the gene encoding for thrombospondin-1 was altered in the brain of WAG/Rij rats, which model absence epilepsy in humans. In addition, we examined the frequency of genetic variants of *THBS1* in a large cohort of children affected by idiopathic/genetic generalized epilepsies (IGE/GGEs).

Methods: We measured the transcripts of thrombospondin-1 and $\alpha_2\delta$ subunit, and protein levels of $\alpha_2\delta$, Rab3A, and the vesicular glutamate transporter, VGLUT1, in the somatosensory cortex and ventrobasal thalamus of presymptomatic and symptomatic WAG/Rij rats and in two control strains by real-time polymerase chain reaction (PCR) and immunoblotting. We examined the genetic variants of *THBS1* and *CACNA2D1* in two independent cohorts of patients affected by IGE/GGE recruited through the Genetic Commission of the Italian League Against Epilepsy (LICE) and the EuroEPINOMICS-CoGIE Consortium.

Results: Thrombospondin-1 messenger RNA (mRNA) levels were largely reduced in the ventrobasal thalamus of both presymptomatic and symptomatic WAG/Rij rats, whereas levels in the somatosensory cortex were unchanged. VGLUT1 protein levels were also reduced in the ventrobasal thalamus of WAG/Rij rats. Genetic variants of *THBS1* were significantly more frequent in patients affected by IGE/GGE than in nonepileptic controls, whereas the frequency of *CACNA2D1* was unchanged.

Significance: These findings suggest that thrombospondin-1 may have a role in the pathogenesis of IGE/GGEs.

KEY WORDS: Thrombospondins, Absence epilepsy, $\alpha_2\delta$ subunit, WAG/Rij rats, Genetic variants.



Ines Santolini is a pharmacologist working on the molecular mechanisms underlying absence epilepsy.

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*I.R.C.C.S. Neuromed, Pozzilli, Italy; †Departments of Neurosciences, Mental Health and Sensory Organs, University Sapienza, Rome, Italy; ‡Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany; §Laboratory of Neurogenetics, “G. Gaslini” Institute, Genova, Italy; ¶School of Pharmacy, University of Lincoln, Lincoln, United Kingdom; #Donders Center for Cognition, Radboud University, Nijmegen, The Netherlands; **Departments of Physiology and Pharmacology, University Sapienza, Rome, Italy; and ††Pediatric Neurology and Muscular Diseases Unit, Departments of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, “G. Gaslini” Institute, University of Genoa, Genova, Italy

Address correspondence to Ferdinando Nicoletti, Department of Physiology and Pharmacology, University Sapienza, Piazzale Aldo Moro, 5, 00185 Rome, Italy. E-mail: ferdinandonicoletti@hotmail.com

¹Co-first Authors.

²For details of the EuroEPINOMICS CoGIE Consortium and Genetic Commission of LICE see Appendix 1.

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KEY POINTS

- Expression of the thrombospondin-1 gene is reduced in the ventrobasal thalamus of a genetic rat model of absence epilepsy
- This reduction precedes the onset of absence seizures, linking thrombospondin-1 to the pathogenesis of absence epilepsy
- Genetic variants of *THBS1* (the gene encoding for thrombospondin-1) are more frequent in patients with IGE/GGE than in healthy controls

Absence epilepsy is a nonconvulsive type of epilepsy characterized by brief periods of unresponsiveness associated with typical spike-and-wave discharges (SWDs) on electroencephalography (EEG). SWDs are generated within a corticothalamocortical network formed by highly excitable neurons in the subgranular layer of the facial region of the somatosensory cortex interconnected with neurons of the reticular and ventrobasal thalamic nuclei.^{1,2} T-type voltage-sensitive Ca^{2+} channels (VSCCs) are critically involved in the generation of SWDs, as demonstrated by the ability of the T-channel blocker, ethosuximide, to normalize the activity of highly excitable cortical neurons and to reduce burst firing of reticular and ventrobasal thalamic neurons.^{3,4}

A growing body of evidence suggests a potential role for the $\alpha_2\delta$ subunit of VSCCs in the pathophysiology of nonconvulsive epilepsy. The mutant mouse *ducky*, which carries a mutation for the gene encoding for the $\alpha_2\delta$ -2 subunit, is characterized by absence seizures and ataxia. In addition, gabapentin and pregabalin, which bind to and negatively modulate $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 proteins, may precipitate absence seizures in experimental animal models and humans.^{5,6} The $\alpha_2\delta$ subunit is known to regulate trafficking, current amplitude, and activation/inactivation kinetics of high-voltage activated VSCCs (e.g., L-, N-, and P/Q-type VSCCs).⁷ The role of $\alpha_2\delta$ subunit in the regulation of T-type channels is less clear. Formation of a multimolecular complex between the α_1 subunit of T channels and auxiliary subunits ($\alpha_2\delta$, β , and γ subunits) has not yet been demonstrated. However, there is evidence that expression of the $\alpha_2\delta$ subunit increases plasma membrane localization and current density of T channels^{8–10} (see also Lacinová et al.,¹¹ for contrasting data). These findings are difficult to reconcile with the evidence that genetic deletion or pharmacologic blockade of the $\alpha_2\delta$ subunit causes absence seizures (see above), unless the function of the $\alpha_2\delta$ subunit is not restricted to the modulation of VSCCs. Of interest, $\alpha_2\delta$ -1 has been identified as a high affinity receptor for thrombospondins,¹² which, in the central nervous system (CNS), are secreted from astrocytes and promote synaptic formation under physiologic and pathologic

conditions.^{13,14} The interaction between the epidermal growth factor-like domains of thrombospondins and $\alpha_2\delta$ -1 mediates the effect of thrombospondins on synaptic formation. Of interest, the $\alpha_2\delta$ ligand, gabapentin, inhibits the induction of excitatory synaptic formation by thrombospondins.¹² This stimulates interest for the study of thrombospondins in models of absence epilepsy.

We used WAG/Rij rats, which develop spontaneous generalized bilateral symmetrical SWDs associated with absence-like behavior after 2–3 months of age.^{15,16} SWDs in symptomatic WAG/Rij rats are reduced by classical antiabsence drugs, such as ethosuximide and valproate, and are increased by drugs that aggravate absence seizures in humans, such as phenytoin, carbamazepine, tiagabine, and vigabatrin.^{17–19} This makes WAG/Rij rats a valuable model for the study of human absence epilepsy.

We now report that the transcript of thrombospondin-1 was largely reduced in the ventrobasal thalamus of WAG/Rij rats, both in the presymptomatic and symptomatic age, as compared to nonepileptic controls. We then examined the variants of *THBS1* and *CACNA2D1* in a cohort of patients with idiopathic/genetic generalized epilepsy (IGE/GGE) and found that variants in *THBS1* but not in *CACNA2D1* were enriched in patients respect to control population. These findings support a potential role for thrombospondins in epileptogenesis and raise the interesting possibility that changes in the expression and/or biologic function of thrombospondin-1 contributes to the pathogenesis of absence epilepsy.

METHODS

Animals

We used male WAG/Rij rats at 2 or 6 months of age raised at Radboud University, Nijmegen, The Netherlands. WAG/Rij rats of 2 months of age do not show SWDs yet, and, therefore, are considered “presymptomatic.” All 6-month-old WAG/Rij rats have about 16–20 SWDs per hour, or >200 SWDs per day,¹⁵ and are thus defined as “symptomatic.” As control rats, we used age-matched *Agouti Copenhagen Irish* (ACI) rats or age-matched Wistar rats. ACI rats show no or only very few SWDs and the lowest number of SWDs of all inbred strains investigated, as assessed in a 48 h EEG evaluation study,²⁰ and in all cases they have much less SWDs than WAG/Rij rats of the same age.²¹ However, ACI rats have a different genetic background with respect to WAG/Rij rats. Wistar rats have the same genetic background of WAG/Rij rats, and may occasionally develop SWDs or have no SWDs at all, depending on the substrain.^{22,23} Rats were housed under a 12 h–12 h light/dark cycle under standard conditions; food, water, and cage enrichment were always available. All efforts were done to cause as little discomfort as possible, and to use as few animals as was considered meaningful. Ethical approval was obtained from Ethics Committee on Animal

Research of the Radboud University Nijmegen (RU-DEC, 'Radboud University Dier Experimenten Commissie'). For the analysis of *Thbs1* and *Cacna2d1* transcripts we performed two independent experiments. In a first experiment, we examined the two transcripts in the ventrobasal thalamus, somatosensory cortex, and motor cortex from WAG/Rij and age-matched ACI rats. In a second experiment, we measured the transcript of *Thbs1* in the ventrobasal thalamus and somatosensory cortex in a new set of 6-month-old WAG/Rij and Wistar rats. For western blot analysis of $\alpha_2\delta 1$, Rab3A, and VGLUT1 protein levels, we compared WAG/Rij, ACI, and Wistar rats for measurements in the ventrobasal thalamus, and WAG/Rij and ACI rats for measurements in the somatosensory cortex.

Biochemical analysis of the principal areas of the corticothalamocortical network

Male WAG/Rij and age-matched control ACI or Wistar rats of 2 or 6 months of age were decapitated, and brains were rapidly dissected out and frozen. The brains from each rat were coded and codes were released after biochemical analysis. Brains were cut coronally by a cryostat, and tissue that comprises the principal area of the corticothalamocortical network was dissected using the coronal diagrams (between coordinates from bregma – 1.88 mm and – 3.80 mm), according to Paxinos and Watson atlas as a guide. For cortical areas, we dissected a part of the cortex that included the primary somatosensory cortex (S1) and another portion of the cortex that included the primary motor cortex (M1). A thalamic portion containing ventrobasal thalamic nuclei (VB) was also dissected.

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from different brain regions of the corticothalamocortical network in Trizol reagent according to manufacturer's protocol. The RNA was further treated with DNase (Qiagen, Hilden, Germany) and single-stranded complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using superscript III (Invitrogen, Carlsbad, CA, U.S.A.) and random hexamers. Real-time polymerase chain reaction (PCR) was performed on 20 ng of cDNA by using specific primers and Power SYBR Green Master Mix (Applied Biosystem, Foster City, CA, U.S.A.) on an Applied Biosystems Step-One instrument. Thermal cycler conditions were as follows: 10 min at 95°C, 40 cycles of denaturation (15 s at 95°C), and combined annealing/extension (1 min at 60°C). Primers used were as follows: *Thbs1* Forw TCGGGGCAGGAAGACTATGA and Rev ACTGGGCAGGGTTGTAATGG; *Cacna2d1* Forw CAGCAATGCTCAGGATGTGA and Rev ATCTGT-TATCCCCTTTGCT; and β -actin Forw GTTGACATCCGTAAAGACC and Rev TGGAAGGTGGACAGTGAG. mRNA copy number of each gene analyzed was calculated

from serially diluted standard curves simultaneously amplified with the samples and normalized against β -actin copy number. Statistical analysis was performed by two-way analysis of variance (ANOVA) and Fisher test.

Western blot analysis

Western blot analysis was carried out in the ventrobasal thalamus and in the somatosensory cortex dissected from WAG/Rij and age-matched control ACI or Wistar rats, at 2 or 6 months of age ($n = 6$ rats per group). Tissue samples were homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin. Proteins were resuspended in sodium dodecyl sulfate (SDS)-bromophenol blue reducing buffer with 40 μ M dithiothreitol (DTT). Western blot analyses were carried out using 8, 10, or 12% SDS polyacrylamide gels, which were electroblotted onto immunoblot polyvinylidene difluoride (PVDF) membranes (BioRad, Milano, Italy); filters were blocked overnight in TTBS (Tris-Tween-Buffer-Saline) containing 5% bovine serum albumin (BSA) or 5% non-fat milk. Specific rabbit polyclonal antibodies for Rab3A (1:1000; Cell Signaling Technology, Danvers, MA, U.S.A.), VGLUT1 (1:1,000; Cell Signaling Technology), $\alpha_2\delta$ -1 (1:200; Sigma, St. Louis, MO, U.S.A.) or mouse monoclonal antibody for β -actin (1:100,000; Sigma) were used. We also used four commercially available anti-thrombospondin-1 antibodies (1:300; Calbiochem, San Diego, CA, U.S.A.; 1:500; Abcam, Cambridge, MA, U.S.A.; 1:500, Thermo Scientific, Rockford, IL, U.S.A.; 1:1000, R&D Systems, Minneapolis, MN, U.S.A.). Blots were then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit or anti-mouse, Amersham, Piscataway, NJ, U.S.A.) diluted 1:7,000 with TTBS. Immunostaining was revealed by enhanced chemiluminescence (ECL, Amersham).

Patients, sequencing, and annotation of the variants

Whole-exome sequencing data from two independent cohorts of IGE/GGE patients recruited through the Genetic Commission of the Italian League Against Epilepsy (LICE) and the EuroEPINOMICS-CoGIE Consortium (Appendix 1) were analyzed. Their clinical information, including data on EEG and antiepileptic therapy, were recorded on data collection forms. Available charts and investigations were reviewed by experienced neurologists (PS, HL, PP) before making a final clinical diagnosis. Genomic DNA isolation and genetic analysis was carried out with the Nimblegen-SeqCapEZ-V244M enrichment kit on the Illumina HiSeq2000 system as described.^{24,25} Only samples with a minimum of 90% of all bases in the coding region of the *THBS1* and *CACNA2D1* being covered by at least 11 reads were used and the Human Gene Mutation Database (HGMD) variants identified in the patients were validated

by Sanger sequencing. Variant annotation was performed using the HGMD Professional 2013. Variant frequencies (minor allele frequency, MAF, >1%) were obtained from the ExAC collection (<http://exac.broadinstitute.org/>). Statistical analysis was carried out by means of two-tailed chi-square with Yates correction.

RESULTS

Reduced thrombospondin-1 gene expression in the ventrobasal thalamus in spontaneously epileptic WAG/Rij rats

We used WAG/Rij rats at 2 and 6 months of age (corresponding to a presymptomatic and symptomatic age, respectively) and age-matched control ACI or Wistar rats for the analysis of thrombospondin-1, $\alpha_2\delta$ -1 subunit, and two biochemical markers of glutamatergic terminals, that is, Rab3A and VGLUT1. All symptomatic WAG/Rij rats show a high frequency of SWDs recorded by EEG as exemplified in Figure 1. *Thbs1* mRNA levels were largely reduced in the ventrobasal thalamus of both presymptomatic and symptomatic WAG/Rij rats, as compared to age-matched nonepileptic control rats (Fig. 2A). Levels did not differ between presymptomatic and symptomatic WAG/Rij rats (Fig. 2A), suggesting that the reduced expression of thrombospondin-1 in the ventrobasal thalamus is not an epiphenomenon of SWDs in WAG/Rij rats. No changes in thrombospondin-1 mRNA levels were found in the somatosensory and motor cortex of WAG/Rij rats at both 2 and 6 months of age (Fig. 2A). To exclude that the observed difference in thalamic *Thbs1* mRNA levels between WAG/Rij and ACI rats was due to the different genetic background of the two strains of rats, we performed an additional experiment in which the transcript of *Thbs1* was measured in the ventrobasal thalamus of WAG/Rij rats and Wistar rats at 6 months of age. Levels were significantly lower in the ventrobasal thalamus of WAG/Rij rats, whereas no changes were found in the somatosensory cortex (Fig. 2B). We were unable to measure thrombospondin-1 protein levels in brain regions of WAG/Rij and control rats because we could not detect a clean band, corresponding to the deduced

molecular size of thrombospondin-1 by western blot analysis, using four commercially available anti-thrombospondin-1 antibodies.

In contrast to thrombospondin-1, no changes in the transcript of *Cacna2d1* were found in the ventrobasal thalamus, somatosensory, and motor cortex of WAG/Rij rats as compared to ACI rats (Fig. 2C). In the somatosensory cortex, the transcript of $\alpha_2\delta$ -1 showed a trend to a reduction with age in both WAG/Rij and ACI rats, whereas a significant increase with age was seen in the motor cortex (Fig. 2C). Immunoblot analysis showed no changes in $\alpha_2\delta$ -1 protein levels in the ventrobasal thalamus of WAG/Rij rats at 2 or 6 months of age, as compared to age-matched ACI or Wistar rats (Fig. 3A). Knowing that thrombospondins enhance the formation of glutamatergic synapses by interacting with $\alpha_2\delta$ -1,¹² we extended the analysis to Rab3A and VGLUT1, which are established presynaptic markers of glutamatergic terminals. Rab3A levels did not change in the ventrobasal thalamus of presymptomatic and symptomatic WAG/Rij rats, as compared to ACI or Wistar rats (Fig. 3B). In contrast, there was a significant strain- and age-related reduction (but not strain \times age difference) of VGLUT1 in the ventrobasal thalamus of WAG/Rij rats as compared to ACI or Wistar rats (Fig. 3C). No changes in $\alpha_2\delta$ -1, Rab3A, or VGLUT1 protein levels were found in the somatosensory cortex of WAG/Rij rats as compared to age-matched ACI rats (Fig. 3D–F).

Genetic variants in the THBS1 gene encoding for thrombospondin-1 are more frequent in IGE/GGE patients

Overall, 238 patients affected by IGE/GGE were included in the analysis (Table S1). One hundred forty-three patients were affected by childhood absence epilepsy (CAE); 21 patients by juvenile absence epilepsy (JAE); 47 patients by juvenile myoclonic epilepsy (JME); and 27 patients by generalized tonic-clonic seizures alone (EGTC). All subjects were of European descent (Italian 128, German 54, Finnish 22, Dutch 11, British 9, Danish 8, Turkish 6). The cohort included 138 female subjects (58%). Age at epilepsy onset ranged from 5 years to 38 years, with a median of 8 years. Most of cases ($n = 183$) derived from multiplex families with at least two affected family members. Most of the patients ($n = 189$; 79.4%) were treated with one antiseizure drug, usually valproate or levetiracetam.

In this cohort, we identified a total of 11 variants (8 distinct HGMD missense mutations in *THBS1* and 3 in *CACNA2D1*) in the whole IGE/GGE cohort (total frequency = 4.62%). Details of the identified variants and their frequency are reported in Table 1. Variants in *THBS1* were enriched in the IGE/GGE cohort (8/238; 3.36%) compared with control population (1829/121,230; 1.5%)

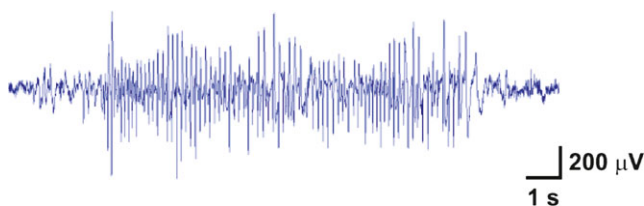
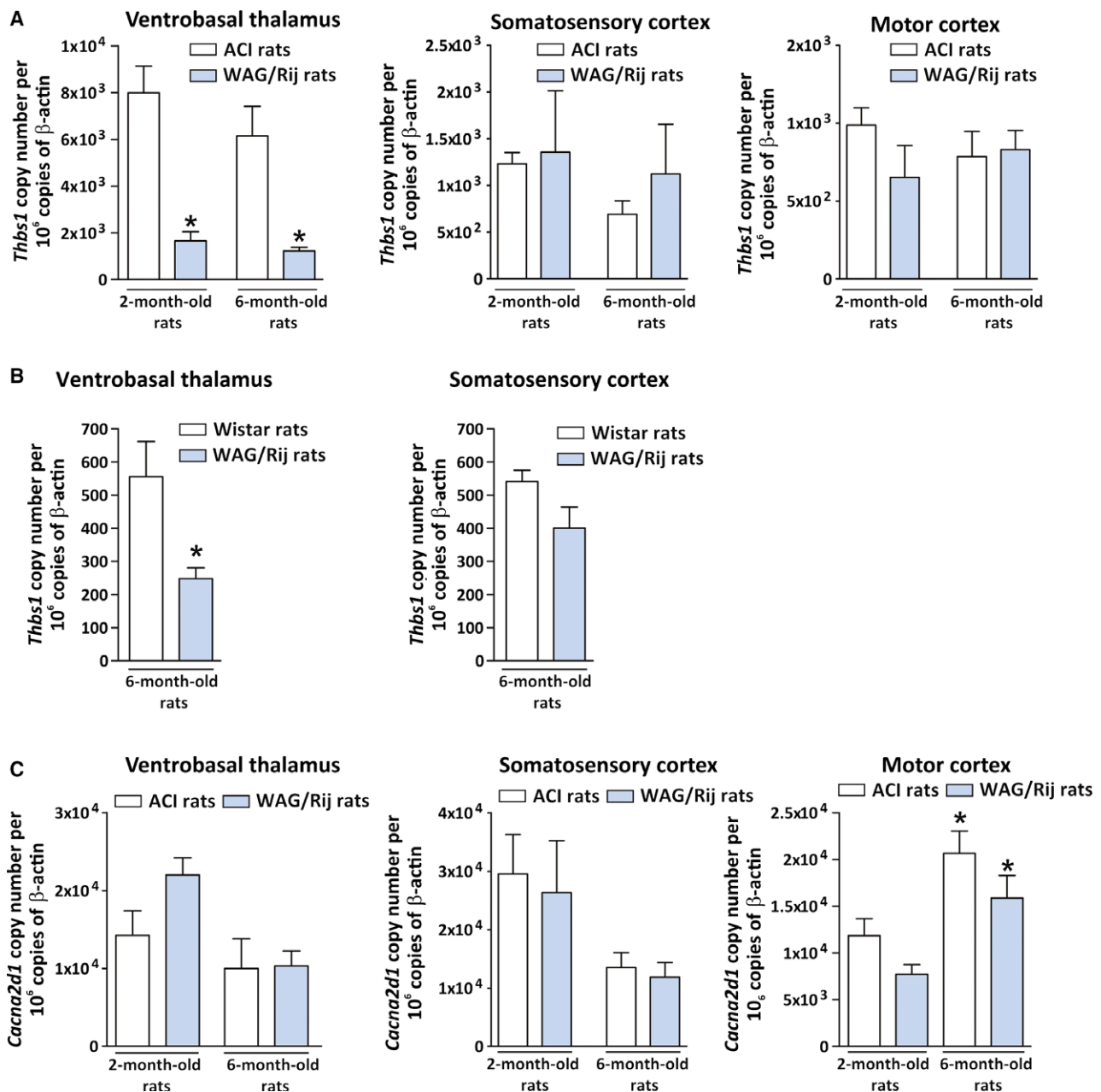


Figure 1. EEG recording showing a typical SWD in symptomatic 6-month-old WAG/Rij rats.
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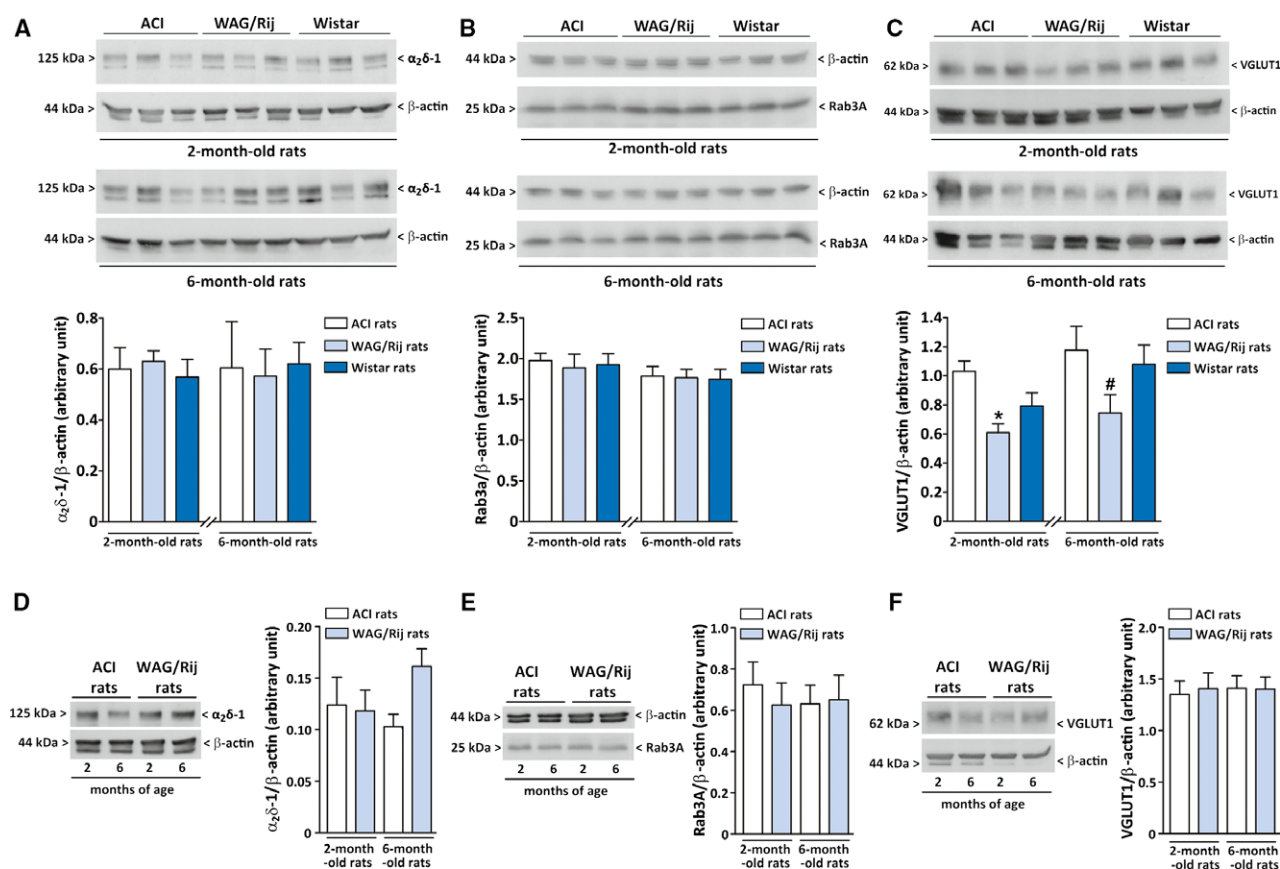
**Figure 2.**

Reduced *Thbs1* transcript in the ventrobasal thalamus of WAG/Rij rats. mRNA levels of *Thbs1* in the ventrobasal thalamus, somatosensory cortex, and motor cortex of WAG/Rij and ACI rats at 2 and 6 months of age are shown in (A). mRNA levels of *Thbs1* in the ventrobasal thalamus and somatosensory cortex of 6-month-old WAG/Rij rats and age-matched Wistar rats are shown in (B). mRNA levels of *Cacna2d1* in the ventrobasal thalamus, somatosensory cortex, and motor cortex of WAG/Rij and ACI rats at 2 and 6 months of age are shown in (C). In (A) and (C), and (B), values are means \pm standard error of the mean (SEM) of 4–5, and 6 animals per group, respectively. In (A), a strain effect for the thalamus was found (two-way ANOVA: $F_{1,15} = 45.65$, WAG/Rij < ACI); post hoc test (Fisher's Least Significant Difference, LSD) confirmed that this strain effect was present for both 2 and 6 months groups * $p < 0.05$. In (B), (Student's t test, $t_{10} = 6.81$, * $p < 0.05$) strain difference in thalamus: 6-month-old WAG/Rij < age-matched Wistar rats. In (C), age effect in motor cortex * $p < 0.05$ (two-way ANOVA, $F_{1,13} = 6.56$, older < younger rats); post hoc test (Fisher's LSD) confirmed that this age effect was present in both strains * $p < 0.05$.

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($p = 0.03$). In contrast, variants in *CACNA2D1* were not more frequent in patients (3/238; 1.26%) than in control populations (733/120,817; 0.6%) ($p = 0.37$) (Table 2).

Syndrome subdivision analysis failed to demonstrate that variants in both genes were specifically enriched in CAE syndrome (Table S2).

**Figure 3.**

Reduced VGLUT1 protein levels in the ventrobasal thalamus of WAG/Rij rats. Western blot analysis of $\alpha_2\delta$ -1, Rab3A, and VGLUT1 in the ventrobasal thalamus of presymptomatic and symptomatic WAG/Rij rats and age-matched ACI or Wistar rats are shown in (A), (B), and (C), respectively. Representative immunoblots from three rats per group are shown. Densitometric values are means \pm SEM of six rats per group. In (C), two-way ANOVA shows a significant strain effect ($F_{2,30} = 7.15$; $p < 0.05$) and age effect ($F_{1,30} = 4.18$; $p < 0.05$), but no strain \times age interaction effect ($F_{2,30} = 0.27$; n.s.). $p < 0.05$ vs. 2-month-old ACI rats (*) or vs. 6-month-old ACI and Wistar rats (#). Western blot analysis of $\alpha_2\delta$ -1, Rab3A, and VGLUT1 in the somatosensory cortex of presymptomatic and symptomatic WAG/Rij rats and age-matched ACI rats are shown in (D), (E), and (F), respectively (densitometric values are means \pm SEM of 6 rats per group).

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DISCUSSION

Thrombospondin-1, one of the five members of the thrombospondin family, is widely expressed in the organism and is known to regulate fundamental cell biologic processes, such as cell attachment to extracellular matrix, cytoskeletal dynamics, and cell migration. In the developing CNS, astrocyte-secreted thrombospondin-1 promotes synaptogenesis, neuronal migration, and axonal growth.^{13,26–29} The demonstration that thrombospondin-1 interacts with the $\alpha_2\delta$ -1 subunit to stimulate the formation of excitatory synapses¹² raised interest on the potential role of thrombospondin-1 in disorders that are targeted by $\alpha_2\delta$ ligands, such as neuropathic pain and epilepsy. Several lines of evidence suggest that an abnormal synaptogenesis contributes to epileptogenesis, that is, to the process by which the brain develops epilepsy. Accordingly, temporal lobe epilepsy in patients and pilocarpine-induced epilepsy in rats

are associated with an increased hippocampal and cortical expression of ephrinB3 and its receptor EphB3, which play a key role in synaptogenesis and mechanisms of synaptic reorganization and plasticity.³⁰ Other proteins that regulate synaptogenesis, such as synapsin II and synaptophysin, have been also implicated in the pathophysiology of epilepsy.^{31,32} Of interest, matrix metalloproteinases (MMPs), which cleave extracellular matrix (EM) proteins and regulate synaptogenesis, have an active role in epileptogenesis in different experimental animal models,³³ and the EM protein, SC1, translocates from neuronal cell bodies to excitatory nerve terminals following status epilepticus in the rat lithium-pilocarpine model.³⁴

Recent evidence suggests that thrombospondin-1, by promoting the formation of new excitatory synapses, contributes to the development of a hyperexcitable neuronal network, which is a critical event in epilepsy. Mendus et al.³⁵ have found that mice lacking thrombospondin-1 or

Table 1. Summary of *THBS1* and *CACNA2D1* variants identified in the IGE/GGE cohort and their frequency from the ExAC collection

Gene	Genomic position	Protein change	MAF	No. minor allele/ No. total alleles	MAF in Europeans	No. minor allele/ No. total alleles in Europeans
THBS1	15:39874505 G/A	p.R60H	0.00004949	6/121,230	0.0000152	1/66,600
THBS1	15:39874642 G/T	p.A106S	NA	NA	NA	NA
THBS1	15:39874742 A/G	p.H139R	0.0001324	16/120,830	0.0001355	9/66,438
THBS1	15:39874918 C/T	p.R198C	0.00003462	4/115,536	0.0000313	2/63,914
THBS1	15:39883456 G/A	p.R773H	0.0001483	18/121,366	0	0/66,698
THBS1	15:39883721 G/A	p.R810Q	0.00002472	3/121,378	0.00002998	2/66,712
THBS1	15:39884907 G/A	p.G891R	NA	NA	NA	NA
THBS1	15:39886304 G/A	p.R1091H	0.0001925	21/109,102	0.0002008	12/59,768
CACNA2D1	7:81588616 T/G	p.D1045A	0.002805	339/120,840	0.004544	302/66,464
CACNA2D1	7:81599241 C/G	p.S755T	0.0007741	93/120,134	0.001209	80/66,170
CACNA2D1	7:81601108 C/T	p.S709N	0.00269	324/120,468	0.004103	272/66,300

MAF, Minor allele frequency. For ExAC collection, see <http://exac.broadinstitute.org/>

thrombospondins 1 and 2 were more sensitive to pentylentetrazole kindling. These mice also showed a reduced expression of $\alpha_2\delta$ -1/2 protein levels in the cortex, suggesting that the thrombospondin/ $\alpha_2\delta$ axis is a key regulator of susceptibility to seizures.³⁵ The importance of the thrombospondin/ $\alpha_2\delta$ axis in the pathophysiology of epilepsy is supported by the evidence that mice overexpressing $\alpha_2\delta$ -1 show epileptiform activity and behavioral arrests associated with an increased number of excitatory synapses in the cortex.³⁶

Here, we showed for the first time that expression of the *Thbs1* gene encoding for thrombospondin-1 was substantially reduced in the ventrobasal thalamus of WAG/Rij rats, which represent an established genetic animal model of spontaneous absence epilepsy.^{15,16} Remarkably, a large reduction in *Thbs1* mRNA levels was observed both in presymptomatic and symptomatic WAG/Rij rats, suggesting that a reduced production of thrombospondin-1 in the thalamus was not secondary to the occurrence of SWDs in

the corticothalamocortical network underlying absence seizures. As opposed to mice lacking thrombospondin-1,³⁵ we did not detect changes in $\alpha_2\delta$ -1 mRNA and protein levels associated with the reduction of thrombospondin-1 in WAG/Rij rats. In contrast, WAG/Rij rats showed lowered VGLUT1 levels in the thalamus with respect to nonepileptic control rats, suggesting that the reduced expression of *Thbs1* gene might have caused an impaired formation of excitatory synapses in the thalamus of WAG/Rij rats.

No changes in THBS-1 gene expression or VGLUT1 protein levels were found in the site of origin of the SWDs, the somatosensory cortex, suggesting that a possible defect in thalamic synaptogenesis combines with a cortical generator in the pathophysiology of absence seizures. The relevance of a defective synaptogenesis in the pathophysiology of absence seizures is supported by the evidence that *stargazer* mice, which show absence seizures, are characterized by an impairment of synaptic formation in the cerebellum,³⁷ which exerts a modulatory role on generalized SWDs through the firing of deep cerebellar nuclei.^{38,39} Double mutant *zi/zi, tm/tm* rats, which represent another rat model of spontaneous absence epilepsy, show a reduced expression of the synaptic vesicle proteins, SV2A and synaptotagmin-1.⁴⁰ A thalamic defect of excitatory synapses may reinforce the hyperpolarizing milieu generated by γ -aminobutyric acid (GABA) released from neurons of the reticular thalamus, thereby facilitating the recovery of T channels from inactivation in ventrobasal thalamic neurons, which ultimately results into pathologic oscillations of the corticothalamocortical network.³ This hypothesis warrants further investigation. Nevertheless, the suggestion that a defect of excitatory neurotransmission is involved in the pathophysiology of absence seizures is supported by the evidence that two types of glutamate receptors, the mGlu1 and mGlu5 metabotropic glutamate receptors, are downregulated in the

Table 2. Statistical analysis of *THBS1* and *CACNA2D1* variants observed in patients affected by IGE/GGE and in subjects from the ExAC collection

	Patients	ExAC collection	Total
<i>THBS1</i>			
Mutated	8	1829	1837
Wild-type	230	121,183	121,413
Total	238	121,230	123,250
% of mutated	3.36	1.5	p = 0.03*
<i>CACNA2D1</i>			
Mutated	3	733	736
Wild-type	235	120,084	120,319
Total	238	120,817	121,055
% of mutated	1.26	0.6	p = 0.37*

*Two-tailed chi-square with Yates correction. For ExAC collection, see <http://exac.broadinstitute.org/>

ventrobasal thalamus of symptomatic WAG/Rij rats, and pharmacologic activation of either mGlu1 or mGlu5 receptors reduces the frequency of SWDs.^{41,42}

IGE/GGEs are the most common types of inherited epilepsy and include at least four well-established epilepsy syndromes, namely, CAE, JAE, JME, and EGTC, all of them characterized by the presence of SWDs/polyspikes.⁴³ Despite its high heritability of 80%, the genetic background is still largely unknown.⁴⁴

To assess the potential impact of thrombospondin and the $\alpha_2\delta$ -1 subunit in the pathophysiology of absence epilepsy in humans, we checked a large cohort of patients affected by IGE/GGE. This analysis revealed that genetic variants of *THBS1* were significantly more frequent in patients affected by IGE/GGE than in nonepileptic controls, whereas the frequency of *CACNA2D1* was unchanged. Syndromic stratification analysis failed to show that variants in both genes were specifically enriched in any of the IGE/GGE syndromes, including CAE, which is in line with the view that IGE/GGE is a spectrum of epilepsy syndromes with variable age at onset associated with common EEG traits generated by pathologic oscillations in a corticothalamocortical network with a cortical origin.³

In conclusion, we showed that expression of thrombospondin-1 gene is reduced in the ventrobasal thalamus of WAG/Rij rats, which model absence epilepsy in humans. This reduction precedes the onset of absence seizures, linking thrombospondin-1 to the pathogenesis of absence epilepsy in WAG/Rij rats. Moreover, a potential role for thrombospondin-1 in the pathogenesis of generalized epilepsy is supported by the finding that polymorphic variants of the *THBS1* gene showed a much higher frequency in a large cohort of patients affected by IGE/GGE compared to control population, although the increase was not significant in the subcohort of patients affected by CAE. Overall these findings support the hypothesis that the thrombospondin-1/ $\alpha_2\delta$ -1 axis is involved in the pathophysiology of epilepsy and encourage further studies on the potential relationship between thrombospondin-1-regulated synaptic formation and epileptogenesis within the corticothalamocortical network.

DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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- Finland; Ann-Kathrin Ruppert, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Auli Siren, Outpatient Clinic for Persons with Intellectual Disability, Tampere University Hospital, Tampere, Finland; Bobby Koeleman, Department of Medical Genetics, Division of Biomedical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands; Dennis Lal, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Felicitas Becker, Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany; Hande Caglayan, Department of Molecular Biology and Genetics, Bogazici University, Istanbul, Turkey; Helle Hjalgrim, Danish Epilepsy Centre, Dianalund, Denmark Institute for Regional Health Services, University of Southern Denmark, Odense, Denmark; Hiltrud Muhle, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany; Holger Thiele, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Ingo Helbig, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany; Janine Altmüller, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Kamel Jabbari, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Kate Everett, Human Genetics Research Centre, St. George's University of London, London, United Kingdom; Patrick May, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg; Peter Nurnberg, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Center for Molecular Medicine Cologne (CMC), University of Cologne, Cologne, Germany; Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany; Rikke Møller, Danish Epilepsy Centre, Dianalund, Denmark; Institute for Regional Health Services, University of Southern Denmark, Odense, Denmark; Rima Nabout, Centre de Référence Epilepsies Rares, Inserm U1129, Neuropédiatries Department, Necker-Enfants Malades Hospital, APHP, Paris Descartes University, CEA, Orsay, France; Roland Krause, Luxembourg Center for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg; Rudi Balling, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg; Stephanie Baulac, Institut National de la Santé et de la Recherche Médicale U975, Centre de Recherche de l'Institut du Cerveau et de la Moelle Epinière, Hôpital Pitié-Salpêtrière, Paris, France; Centre National de la Recherche Scientifique, Paris, France; Université Pierre et Marie Curie (Paris VI), UMR_S 975, Paris, France; Thomas Sander, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Wolfram Kunz, Department of Epileptology and Life & Brain Center, University of Bonn Medical Center, Bonn, Germany; Yvonne Weber, Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany.

Collaborative Group of Italian League Against Epilepsy (LICE) Genetic Commission

Amedeo Bianchi, Angela La Neve, Antonietta Coppola, Salvatore Striano, Giuseppe Capovilla, Edoardo Ferlazzo, Irene Bagnasco, Alessandro Ferretti, Pasquale Parisi, Carlo Di Bonaventura, Maria Stella Vari, Francesca Pinto, Francesca Bisulli, Paolo Tinuper, Carlo Minetti, Vincenzo Belcastro, Lucio Giordano, Antonio Gambardella.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical data overview of the investigated IGE/GGE cohort.

Table S2. Statistical analysis of *THBS1* and *CACNA2D1* variants observed in CAE patients and in controls from the ExAC collection (<http://exac.broadinstitute.org/>).

APPENDIX I

EuroEPINOMICS CoGIE Consortium

Aarno Palotie, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom; Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland; Program in Medical and Population Genetics and Genetic Analysis Platform, The Broad Institute of MIT and Harvard, Cambridge, MA, U.S.A.; Anna-Elina Lehesjoki Folkhälsan, Institute of Genetics, Helsinki, Finland; Research Programs Unit, Molecular Neurology, University of Helsinki, Helsinki, Finland; Neuroscience Center, University of Helsinki, Helsinki,